Virulence-specific cell cycle and morphogenesis connections in pathogenic fungi

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Running title: Virulence and cell cycle in fungi

Abstract

To initiate pathogenic development, pathogenic fungi respond to a set of inductive cues. Some of them are of an extracellular nature (environmental signals), while others are intracellular (developmental signals). These signals must be integrated into a single response whose major outcome is changes in the morphogenesis of the fungus. The regulation of the cell cycle is pivotal during these cellular differentiation steps; therefore, cell cycle regulation would likely provide control points for infectious development by fungal pathogens. Here, we provide clues to understanding how the control of the cell cycle is integrated with the morphogenesis program in pathogenic fungi, and we review current examples that support these connections.

1. Introduction

In metazoans, cell division and cell differentiation are intimately intertwined [1]. These processes substantially overlap during the development of any pluricellular organism. By controlling the cell cycle, developmental signals determine cellular morphogenesis, which defines new cell types. However, sometimes it is cell cycle regulation that determines whether a cell is able to perceive the developmental signals for differentiation. In addition, terminal differentiation at the end of a particular developmental program is often characterized by permanent withdrawal from the cell cycle, and therefore pathways controlling exit or entry into the cell cycle have dramatic consequences on the ability of a cell to differentiate.

In contrast, it was thought for many years that cell cycle regulation had little effect on the ability of a fungal cell to differentiate [2]. There were several reasons for this belief. One important reason was that, with the exception of terminal quiescent spores, cell cycle withdrawal is rare during the morphogenesis of specialized structures in fungal cells. In addition, primary studies of fungal cell cycle regulation were performed in budding and fission yeasts, both with very limited developmental options. Today, this scenario is changing, and recent studies of the influence of cell cycle regulation on the ability of pathogenic fungi to infect their hosts are paving the way for new understanding [3-6].

Pathogenic fungi are excellent systems in which to study developmental choices in simple eukaryotes. The activation of the virulence program requires the integration of both environmental signals (nutrient availability, temperature, host signals and others) as well as internal cues (metabolic status, mating types and others). One of the major outcomes of the activation of the virulence program is the morphogenesis of the fungus to produce specific structures that help the process of infection [7]. To date, the primary experimental approaches used to define and study the regulation of the pathogenic developmental programs in fungi have been focused on studying signal transduction and transcriptional changes. However, during the last decade, novel opportunities have become available to investigate the molecular basis of fungal pathogenicity from a novel point of view that is complementary to previous approaches in the field [8]. The main premise of these studies was to assume that there are novel roles for cell cycle and morphogenetic regulators in pathogenic fungi: roles that may help adapt the cell to the virulence program. Clearly, the cell cycle and morphogenesis machineries are attractive targets through which signaling may coordinately regulate fungal morphogenesis and cell-cell interactions, and thereby virulence [6].

In this review, we will examine the connections between cell cycle regulation and morphogenesis in fungi, as well as summarize recent studies that have investigated these connections during the induction of the virulence program in pathogenic fungi.

2. Cell cycle and morphogenesis: clues

A complete eukaryotic cell cycle is composed of four phases: the synthesis (S) phase, the mitotic (M) phase, and the two intervening gap phases, G1 and G2. The engine that drives the switch-like transitions between the distinct phases consists of a protein heterodimer complex containing a cyclin and an associated kinase moiety. This group of kinases is referred to as the cyclin-dependent kinases (CDKs). In fungi, a single kinase called Cdk1 (Cdc28 in *Saccharomyces cerevisiae*) drives the cell cycle. The stage-specific activities of Cdk1 are determined by interacting with different phase-specific cyclins [9]. In terms of the cell cycle, the more important phases are the S phase, during which the DNA is replicated, and the M phase, during which the replicated genetic material is segregated into the two daughter cells. However, during each cell cycle, eukaryotic cells attain specific morphologies in addition to replicating and segregating the genome. In this sense, distinct growth patterns are regulated during the gap phases G1 and G2 (Fig. 1A).

In fungi, for which the morphology is determined by cell wall formation, there are two main patterns of growth: a localized cell wall expansion at the growth tip (polarized growth) or a uniform cell wall expansion over the cell surface (isotropic growth). The first type of growth will produce elongated cells, while the second one will produce round cells. Changes in the balance of polarized versus isotropic growth can generate the variety of cell shapes observed during fungal development [2].

Insights into the links between cell morphology and cell cycle regulation in fungi have primarily come from studies of the budding yeast *S. cerevisiae* [10]. The budding yeast cell cycle has two growth pattern switches: bud emergence at the end of G1 phase upon polar growth (directed toward the bud tip) and a

subsequent switch to isotropic growth (uniform bud expansion) during the G2/M phase, resulting in round daughter cells. The switch between polar and isotropic growth is controlled by the activity of the distinct CDK complexes. Two main classes of cyclins can be distinguished with respect to their effects on growth pattern: G1 cyclins (Cln) and G2 or mitotic cyclins (Clb). The distinction between the G1 and G2 types of cyclins is pertinent to morphogenesis because, as discussed below, Cdc28/Cdk1 associated with G1 cyclins (CDK^{G1}) promotes polar growth, while Cdc28/Cdk1 associated with G2 cyclins (CDK^{G2}) promotes isotropic growth by suppressing polar growth [11]. Overexpression of Cln cyclins (increasing CDK^{G1} activity) or decreasing the levels of Clb cyclins (low CDK^{G2} activity) results in the inhibition of the switch from polar to isotropic growth, and the buds become elongated and polarized [12-14]. Therefore, depending on the activity of each CDK class at different phases, one growth pattern is selected (Fig. 1B).

The morphogenetic activity of CDK^{G1} in *S. cerevisiae* relies on the regulation of the highly conserved GTPase Cdc42. This Rho-like protein acts as a GTP-regulated molecular switch that is ‘on’ in the GTP-bound state and ‘off’ when GDP-bound [15]. Cdc42 triggers morphology-related events, such as cytoskeletal remodeling, polarized secretion, and endocytosis, in its ‘on’ version. However, proper morphogenesis requires cycling between the GTP-bound and GDP-bound states. This cycling is controlled by GAPs (GTPase activating proteins), which enhance nucleotide hydrolysis (resulting in the ‘off’ state), and GEFs (guanine exchange factors), which catalyze nucleotide exchange (resulting in the ‘on’ state). CDK^{G1} activates Cdc24, the sole GEF of Cdc42 in *S. cerevisiae* [16], while at the same time inhibiting the cognate GAPs Rga2, Bem2

and Bem3 [17, 18]. This way, CDK^{G1}-mediated phosphorylation events enhance the ability of the cell to undergo polarized growth. Although this control seems to be the primary mechanism, additional morphogenetic targets for CDK^{G1} exist, and these mechanisms apply to other pathways beyond Cdc42. For instance, CDK^{G1} regulates Tus1, a GEF for Rho1, another GTPase to promote cell polarization [19].

In opposition to CDK^{G1}, CDK^{G2} activity inhibits apical growth and triggers isotropic growth. More than negatively controlling the activity of Cdc42 (which simply abrogates growth), it seems that in *S. cerevisiae* the rise in CDK^{G2} activity results in the redistribution of Cdc42 and Cdc24 over the whole bud cortex so that the actin cytoskeleton becomes a diffuse unpolarized network of actin cables [13]. However, the molecular targets of CDK^{G2} that mediate these events are poorly known. Recently, it was described that CDK^{G2} mediates a complex phosphorylation process acting on Lte1, a GEF involved in the activation of the MEN cascade, the main promoter of cytokinesis. Upon CDK^{G2} phosphorylation, Lte1 becomes a small GTPase inhibitor, gaining high affinity for the small GTPases Ras1 and Bud1 in a hitherto unknown process that prevents polarized growth [20].

How general is the CDK-morphogenesis connection in other fungal systems? Many key components of the Cdk1 and Cdc42 pathways are conserved among fungi, suggesting that this regulation may apply to growth patterns in other species. Yeast cells that produce filamentous growth (dimorphic yeast) are excellent systems to study these connections. In the dimorphic yeasts *Candida albicans* (a human pathogen) and *Ustilago maydis* (a plant pathogen), CDKs also play important roles in morphogenesis during both yeast and hyphal

growth. In contrast to the redundancy observed in *S. cerevisiae* (three G1 (Cln1–3) and six G2 (Clb1–6) cyclins), *C. albicans* and *U. maydis* have only two G2 cyclins (Clb2 and Clb4 or Clb1 and Clb2, respectively) [21, 22]. There are two G1 cyclins (Ccn1 and Cln3) in *C. albicans* [23-25] and a single G1 cyclin (Cln1) in *U. maydis* [26]. Altering the levels of each cyclin results in dramatic morphological changes. For example, decreasing the CDK^{G2} activity by depleting G2 cyclins results in strong polar growth in both yeasts, while increasing the activity of CDK^{G2} via the overexpression of G2 cyclins disables the ability to produce filamentous growth [21, 22]. Furthermore, in *U. maydis*, the overexpression of the Cln1 G1 cyclin results in strongly polarized cells, while deletion results in aberrant round cells [26]. These examples support the hypothesis that, as described in *S. cerevisiae*, CDK^{G1} promotes polar growth and CDK^{G2} inhibits polar growth promoting isotropic growth in other fungal systems. Furthermore, studies in both dimorphic yeasts also suggested that the connections between CDK^{G1} and Rho-like GTPases observed in *S. cerevisiae* could also be conserved. In *S. cerevisiae*, Cdc42 is solely responsible for the regulation of polar growth and cytokinesis. However, in other fungi, two distinct Rho GTPases, Rac1 and Cdc42, are devoted to polar growth and cytokinesis. Surprisingly, the degree of the deployment of Cdc42 and Rac1 for critical morphogenetic functions seems to vary even among related fungi [27]. For instance, while Cdc42 is devoted to cytokinesis and Rac1 is specific for polarized growth in *U. maydis* [28], in *C. albicans* both GTPases are required for filamentous growth, depending on the stimulus [29]. Interestingly, in *C. albicans*, CDK^{G1} phosphorylates Rga2 (which acts as a GAP of Cdc42) upon induction of highly polarized hyphal growth [30]. This modification results in the removal of

Rga2 from the hyphal tip to allow proper growth and morphology. In a similar way, in *U. maydis* an alternative CDK^{G1} (Cdk5, see below) regulates the association between the scaffold protein Bem1 and Cdc24, which in *U. maydis* acts as a GEF of Rac1, and thereby promotes polar growth [31]. Moreover, studies in pathogenic fungi will uncover new connections between CDK activity and morphogenesis. For instance, in *C. albicans*, Mob2 (the regulatory subunit of a well-conserved NDR kinase with morphogenetic roles) must be phosphorylated by Cdc28 during hyphal growth [32].

In summary, it seems that during fungal morphogenesis, the balance between two opposing cyclin-dependent activities (CDK^{G1} and CDK^{G2}) determines the equilibrium between polar and isotropic growth, which determines the cell shape. In a naïve way, to describe the connections between the cell cycle and morphogenesis, it is fair to say that to obtain sustained polar growth, we have to provide the cell with high CDK^{G1} activity and at the same time turn down CDK^{G2} activity, while for isotropic growth turning on CDK^{G2} (which has an inhibitory role in polar growth) should be enough. Therefore, how the respective CDK activities are regulated may have a dramatic influence on cell morphology. In general, cyclins are synthesized periodically prior to the stage at which their activity is required and are degraded thereafter. Such periodic oscillation ensures unidirectional cell cycle progression, and synthesis and specific degradation are among the main ways to regulate the activity of each CDK complex. In terms of morphogenesis, that denotes that polar growth will appear only during G1 (where CDK^{G1} will be present and CDK^{G2} absent) and that during G2 (where CDK^{G2} will be present), only isotropic growth is possible. In fact, this is similar to the process in *S. cerevisiae*. However, for a huge number of fungal species,

sustained polar growth during both the G1 and G2 phases is normal. How is this possible?

With respect to CDK^{G1} activity, given that the presence of G1 cyclins is limited to the G1 phase, the ability of the cell to keep polar growth out of the G1 phase is assured by the use of morphogenesis-specific G1 cyclins. This is the case for the *C. albicans* Hgc1 cyclin, which is expressed in response to filamentation inducers and promotes strong polar growth [33]. In other cases, CDK^{G1} activity is provided by alternative catalytic CDK subunits, such as the kinases belonging to the Pho85 family [34]. Moreover, in some cases, such as Cdk5 in *U. maydis* [35] or PhoA and PhoB in *A. nidulans* [36] these kinases are essential for polar growth. These kinases interact with alternate G1-like cyclins (Pcl cyclins) that are often very specific for a process. For example, in *U. maydis,* the promotion of polarized growth during the formation of the infective hyphae requires the specific expression of the alternative cyclin Pcl12, which interacts with Cdk5 [37]. In *C. albicans,* the Pcl1/Pho85 regulatory pair is involved in temperature-dependent filamentation [38] (Fig. 1B).

As polar growth also requires down-regulation of CDK^{G2}, in addition to out-of-phase G1-like cyclin expression, there must be ways to suppress the CDK^{G2} complexes to assure polar growth during the G2 phase. One of these ways is through the phosphorylation of the catalytic Cdk1 subunit in a highly conserved tyrosine residue at the N-terminus. This inhibitory phosphorylation is produced by the Wee1 family of protein kinases and can be counteracted by the action of Cdc25 phosphatase family members [39]. In fungal cells, this negative regulation is exclusive of CDK^{G2} complexes. In a regular cell cycle, the balance between Wee1 and Cdc25 activities determines the activity of CDK^{G2}

complexes and thereby determines the entry into mitosis as well as morphogenesis. High levels of Wee1 activity or downregulation of Cdc25 activity result in a net inhibition of CDK^{G2} complexes, promoting polar growth [40, 41]. Upstream of Wee1 and Cdc25, there are a plethora of regulators that help to integrate distinct signals into the cell cycle and thereby into morphogenesis. The Wee1 kinase is negatively controlled by a family of kinases (Nim1-like family), which has a dramatic effect on morphogenesis. Cells defective in Hsl1 (one of the Nim1-kinases) are elongated in at least three different species: *S. cerevisiae*, *C albicans* and *U. maydis* [42-44]. Nim1-like kinases integrate nutritional, morphogenetic and developmental cues into the cell cycle and morphogenesis processes. The Cdc25 phosphatase is also regulated by upstream components, some of which are related to the response to DNA damage, such as Chk1 kinase [45] (Fig. 1B).

3. Cell cycle and morphogenesis: consequences

How cell cycle regulation affects cell morphology is clearly exemplified by comparing *S. cerevisiae* and *U. maydis*. Both yeasts divide by budding; however, while *S. cerevisiae* cells are round, *U. maydis* cells are elongated and have a cigar shape. The different morphologies reflect different contributions of polar and isotropic growth patterns during the distinct phases of the cell cycle. In *S. cerevisiae*, polar growth is confined to the G1 phase and the cell switches to isotropic growth during G2, while in *U. maydis,* polar growth occurs in the G1 phase and reaches its apex during the G2 phase. The reason for this distinct growth pattern is related to the extent of the Wee1-mediated inhibitory phosphorylation of CDK^{G2} complexes that each yeast experiences. In *U. maydis*,

Wee1 remains active along the G2 phase and is counteracted by Cdc25 only when cells enter mitosis [4], while in *S. cerevisiae*, Swe1 (the Wee1 ortholog) is degraded at the beginning of the G2 phase, resulting in no inhibitory phosphorylation during this stage [46]. One of the outcomes of this differential regulation is the distinct bud morphology. In *U. maydis*, the emersion of the bud starts as soon as the cell reaches G2 phase and the cell undergoes strong polar growth during this period, coincident with high Wee1 activity (low CDK^{G2} activity). In contrast, in *S. cerevisiae,* the budding process starts at the end of the G1 phase upon the activation of polar growth directed toward the bud tip. This growth subsequently switches to isotropic expansion during G2 because of the lack of negative control over CDK^{G2} activity. In fact, mutant cells in which the Swe1 protein is not degraded form elongated buds [42].

The most obvious consequence of the connections between the cell cycle and morphogenesis is that by controlling the activity of the distinct CDKs, it is possible to determine the morphology of the cell. This is particularly important for pathogenic fungi because in order to breach the barriers faced by their hosts, they have to produce specialized structures, which are sometimes very specific for each fungal species. Therefore, it is expected that activation of the virulence program will affect the regulation of the cell cycle to allow the formation of specific infection structures, among other processes.

4. Exploring the surface: activating polar growth

Many fungi initiate their life cycle with the germination of spores. During the process of germination, a short germ tube emerges and grows into a hypha

capable of impressive extension rates. In the case of pathogenic fungi, particularly plant and insect pathogens, this hypha, the so-called infective filament, serves to explore the host surface and search for an entry site into the host tissue. Eventually, depending on the fungus, this infective hypha will differentiate into specific structures required to penetrate the host tissue [7]. For some mammalian pathogens in which the infective phase involves spores that are inhaled by the host, such as *Aspergillus fumigatus* or *Cryptococcus neoformans*, the ability to germinate and to form a filament determines the success of the infective process. In some mammalian pathogens, such as *C. albicans*, the ability to rapidly produce a filament is crucial to escape from the immune system. The mammalian immune response against human fungal pathogens relies mainly on phagocytosis of the fungus by cells of the innate immune system. Once the fungus has been engulfed by the macrophage, strong hyphal growth of the fungus leads to associated macrophage lysis and escape [47].

In other words, the ability to strongly activate polar growth at the correct time is crucial for infection in a number of fungal pathogens. As discussed above, in order to promote polar growth, the cell requires high CDK^{G1} activity and low CDK^{G2} activity. Interestingly, the characterization of a very few cases has indicated that there are distinct ways in which this can be achieved.

One way to promote polar growth is to circumscribe the formation of the infective hypha to the G1 phase, elongating this phase until the infective hypha reaches the correct size for differentiation into an infection-related structure. This is how *Colletotrichum orbiculare*, the causal agent of cucumber anthracnose disease, produces an infective hypha [48]. The infection process

initiates with the germination of aseptate conidia, which results in a germ tube that eventually differentiates into a specialized dome-shaped structure called the appressorium, required for host penetration. Interestingly, during conidial germination, which requires the establishment of strong polar growth to produce the germ tube, there is a delay in G1/S progression, most likely to sustain the emersion of the germ tube. This cell cycle delay involves a two-component GAP complex (Bub2/Bfa1), which apparently targets the GTPase Tem1. Lack of function mutants in either GAP component result in a premature G1/S transition and a delay in the emersion of the germ tube, as well as additional posterior defects that fatally impair the infective process [48]. How the Tem1 GTPase is able to control the G1/S transition in this fungus is unknown, taking into account that in other systems these proteins are involved in mitosis exit [49]. Additionally, the manner in which the virulence program controls this delay in G1/S transition is unknown.

Another way to promote polar growth is to inhibit CDK^{G2} activity, which may result in the inability to produce the G2/M transition and therefore the cells will remain arrested in the G2 phase, and at the same time to provide some alternative CDK^{G1} activity. This is essentially the method that *U. maydis* uses to produce its infective filament. In this fungus, the infective filament does not result from the germination of a spore but is produced as a result of a mating process between two compatible yeast-like cells. The establishment and maintenance of the infective filament is controlled by a heterodimeric transcription factor, the b-complex, with the subunits provided by each compatible mating partner, and it is assembled after cytoplasm fusion. The b-complex activates a transcriptional cascade that, among other actions, induces

the arrest of the cell cycle at G2 and activates strong polar growth. As a result, a dikaryotic infective filament is produced that, when growing on the plant surface, is composed of a single arrested cell, which is able to grow up to 100 μm [3]. This strong polar activity is supported by a dramatic increase in CDK^{G1} activity. Upon b-complex formation, there is an enhancement of the transcription of *pcl12*, which encodes an alternative G1-like cyclin that interacts with its cognate Cdk subunit, Cdk5. The activity of the $Cdk5^{Pcl12}$ complex dramatically enhances the polar growth of the infective filament [37, 50]. To down-regulate CDK^{G2} activity, the virulence program promotes an imbalance in the activities of the kinase Wee1 and the phosphatase Cdc25, resulting in a net increase in the level of CDK^{G2} inhibitory phosphorylation [51]. Some of the molecular details describing the process are well understood. The b-complex negatively regulates the expression of *hsl1*, encoding a Nim1-family kinase that is a negative regulator of Wee1. In this way, the activity of Wee1 is exacerbated [43]. At the same time, the b-complex activates the DNA damage response (DDR) cascade composed of the kinases Atr1 and Chk1, resulting in the inhibition of the Cdc25 phosphatase [51, 52]. The use of the DDR cascade to promote polar growth by virtue of its ability to down-regulate CDK^{G2} complexes seems to be more frequent than expected in the fungal world. It has also been described in the filamentous yeast *Schizosaccharomyces japonicus* [53], as well as in *C. albicans* [54]. In the case of *U. maydis*, how a transcription factor such as the b-complex activates the DDR cascade is unknown, but it does not appear to require the presence of damaged DNA [55].

The study of the induction of filamentation in *C. albicans* provides a third example of alternative methods to manipulate CDK activity to enhance polar

growth. Interestingly, in this organism, cell cycle transitions are independent of hyphal elongation [56]. In contrast to the examples described above, during filamentation, the presence of CDK^{G2} activity does not impair the ability of the cell to produce polar growth; hyphal extension continues throughout mitosis, during which CDK^{G2} activity reaches its apex [57]. The reasons for this apparent independence of the cell cycle are related to the expression of a G1 cyclin called Hgc1, which specifically functions to promote hyphal growth. Unlike the cell cycle-regulated expression of other cyclin genes, the transcription of *HGC1* is strongly activated at any cell cycle stage by hyphal-inducing signals [33]. A study of the polarity targets of $Cdc28^{Hgc1}$ revealed the expected relationships, such as the Cdc42 GAP Rga2 [30], but also new connections, such as the exocyst, which is required for proper secretion. While in *S. cerevisiae* the exocyst subunit Exo84 is phosphorylated by $Cdc28^{Clb2}$, causing the exocyst complex to disassemble [58]; in *C. albicans*, Exo84 is phosphorylated by $Cdc28^{Hgc1}$, and this phosphorylation is necessary for the efficient polarized growth of hyphae [57]. In other words, the expression of Hgc1 overwhelms the activity of the CDK^{G2} complexes to oppose the maintenance of polar growth thanks to alternative wiring between cell cycle regulators and the elements required for proper polar growth. In this way, only the presence of specific CDK^{G1} activity is required to promote polar growth. It is worth noting that *C. albicans* is able to grow in a variety of cellular forms and that the ability to change shape is exploited by the fungus to promote virulence and to escape from the immune system [59]. Most likely, the evolution of this alternative control for ensuring cell cycle-independent hyphal extension allows *C. albicans* to activate changes in morphology regardless of the cell cycle phase and at the

same time keeping proliferation active (i.e., it does not involve cell cycle arrest or delay for morphological changes).

5. Invasion tools: appressorium formation and cell cycle regulation

To breach the plant cuticle, which represents a primary barrier in the defense against pathogens, phytopathogenic fungi often differentiate into specific infection structures termed appressoria. The morphology of the appressoria is highly variable, most likely reflecting distinct genetic programs in different fungi [60]. However, despite this diversity in form and function, all appressoria formations known require readjustment of the cell cycle to allow the induction of these new morphogenetic programs. To illustrate how different appressorium morphologies require distinct cell cycle alterations, we will compare two extreme cases of appressorium formation. The appressorium made by *Magnaporthe oryzae* is a dome-shaped structure with a thick, multilayered and highly melanized cell wall that penetrates the plant using the turgor pressure produced inside the fungal cell [61]. The appressorium produced by *U. maydis* is difficult to distinguish morphologically because it represents only a slight swelling of the germ tube apex that directs the localized secretion of enzymes that weaken the plant cuticle and cell wall [62].

Appressorium formation in *M. oryzae* starts with the germination of one of the cells composing the three-celled conidium. During this process, a germ tube is produced with a single nucleus at G1, and by analogy with *C. orbiculare*, it is likely that an active delay of the G1/S phase occurs to allow polar growth. Once

the germ tube reaches the proper size (approximately 15 μm), an incipient appressorium is initiated by isotropic expansion at the germ tube tip. This isotropic expansion most likely requires that the cell enters the G2 phase (high CDK^{G2}), given that when germinating conidia are treated with the DNA replication inhibitor hydroxyurea (HU) or when thermo-sensitive mutants for the initiation of DNA replication are incubated at semi-restrictive temperature, appressorium formation does not occur and instead the conidia develop elongated germ tubes [63]. Once the appressorium expands, it needs to mature, which requires the cell nucleus to enter mitosis. When thermo-sensitive mutants in the protein kinase NimA, which is required to activate mitosis, are incubated at a semi-restrictive temperature, appressorium maturation is prevented. The requirement of mitosis for maturation most likely is related to the necessity to stop cell wall expansion (cessation of cell growth during mitosis is a common feature among eukaryotic cells). The maturation process involves, among other things, the melanization of the appressorium, the generation of internal turgor pressure, and the deposition of cytoskeletal components at the base of the appressorium pore to allow the formation of the penetration peg, where all the turgor pressure will be focused [64]. As mitotic growth arrest is thought to help cells to reorganize their structure and adapt to the increased energy demands needed for subsequent cell division or regeneration [65], it makes sense to stop cell wall expansion in the appressorium to allow maturation to proceed. The next step during the formation of a functional appressorium is the remodeling of the F-actin cytoskeleton to form a toroidal network at the base of the appessorium, which is organized by septins. This marks the point of penetration peg emergence [64]. Most likely, this reorganization requires removal of the

CDK^{G2} activity, since preventing the progression of mitosis and exit of the cell cycle, either through a thermo-sensitive mutant in the anaphase-promoting complex or using a stabilized version of a G2 cyclin, Cyc1, results in a mature melanized appressorium that is not functional and is unable to infect the plant [66].

In contrast with the distinct steps observed in *M. oryzae*, the appressorium of *U. maydis* is relatively simple. The infective filament is composed of a single cell that is arrested at the G2 phase, which expands by apical growth. Eventually, the infective filament will differentiate into an appressorium and it will penetrate the plant tissue, where cell cycle arrest will be released and proliferation will resume. The appressorium is produced in response to unidentified plant signals, and it consists of a small swelling of the hyphal tip that is unmelanized and points to the plant surface [67]. Appressorium formation results in a localized area of secretion where plant cell wall-degrading enzymes, which help penetrate the cuticle [62], and specific effector proteins required for the precise signaling occurring during infection [68] are concentrated. During this process, polar growth is sustained, and appressoria simply mark the point at which the growth direction changes. Importantly, an inability to maintain the G2 cell cycle arrest results in a lack of appressorium formation [43, 69]. Why is sustaining G2 cell cycle arrest important during appressorium formation? In *U. maydis*, as in other systems, entry into mitosis demands the recruitment of a large quantity of cytoskeletal elements to form the mitotic spindle [70]. In addition, support for localized secretion most likely depends on the coordinated use of both actin- and microtubule-based cytoskeletons. Therefore, it is likely that mitosis and the morphogenetic program responsible for appressorium formation compete for

the same cytoskeletal components. If this is the case, it makes sense that cellular controls exist to force these two processes to be incompatible. In fact, this incompatibility is reflected by the ability of CDK^{G2} activity to phosphorylate and inactivate the transcriptional regulator Biz1 (Pérez-Martín, unpublished data), a transcriptional factor that is required for activation of the transcriptional program that develops the appressorium in *U. maydis* [71].

As described above, one clear difference between *M. oryzae* and *U. maydis* appressoria occurs at the maturation step. For *M. oryzae,* penetration peg development requires coupling with mitosis, leaving one daughter nucleus at the appressorium and the other traveling with the penetration peg [66]. However, in *U. maydis,* the cell cycle is not reactivated until the infective dikaryotic hyphae penetrate the plant tissue. In this case, the two genetically distinct nuclei travel at the tip of the filament [67]. This uncoupling between mitosis and penetration in *U. maydis* is probably a consequence of the peculiarities of the complex cell cycle required to maintain heterokaryosis after cell division. In some basidiomycetes, as is the case for *U. maydis* and *Coprinopsis cinerea*, nuclear division involves the production of a specific structure called the clamp-like cell [72-74], devoted microtubule structures [75], and the activation of a specific checkpoint controlled by the DDR pathway [52, 72]. Again, it makes sense that during the penetration step, which in *U. maydis* is apparently not dependent on turgor pressure but on continuous communication between the plant and the fungus that involves the dedicated secretion of effector proteins, mitosis has to be delayed.

6. Concluding remarks

Many of the relationships between the cell cycle and morphogenesis in pathogenic fungi have a high degree of plasticity. An emerging theme is that cell cycle and morphogenesis connections are species specific. Thus, there is no single mechanism but instead many different modes that connect the cell cycle and morphogenesis, which are adapted for various settings that mainly depend on the specific wiring of the distinct, but otherwise conserved, cell cycle and morphogenesis regulators. Understanding these connections is clearly one of the grand challenges in fungal developmental biology for the next decade or more. Currently, we are beginning to unravel these intricate relationships. We can only base our knowledge on detailed studies of very few examples. However, new systems to study these relationships are on the horizon. These include the plant pathogen *Fusarium graminearum*, in which two distinct Cdk1 kinases are active during the vegetative and infectious phases [76], and the entomopathogenic fungus *Beauveria bassiana*, in which virulence seems to be controlled by the level of Cdk1 inhibitory phosphorylation [77]. There is an exciting journey of discovery awaiting the discipline.

Acknowledgements

We thank Prof. J. Correa-Bordes (Universidad de Extremadura) and Prof. N. J. Talbot (University of Exeter) for critical reading of the manuscript. We also thank Prof. Correa-Bordes for inspiring discussions along the writing of this review. Research in the Pérez-Martín laboratory is supported by grants from the Spanish Government (BIO2014-55398-R) and ITNs from the EU (Ariadne and Fungibrain).

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Legends to the Figures

Fig. 1. Connections between cell cycle and morphogenesis: clues.

(A) Scheme of a core eukaryotic cell cycle. It comprises four phases: the synthesis (S) phase, the mitotic (M) phase, and the two intervening gap phases, G1 and G2. During S phase, the DNA (schematized as a single chromosome) is replicated, whereas during M phase, the replicated genetic material is segregated into the two equivalent DNA copies. The core engines that drive the progression through the eukaryotic cell cycle are the CDK complexes, which accumulate during the respective gap phases and triggers the G1/S and G2/M transitions.

(B) Effects of the distinct CDK complexes with respect to the choice of growth. Different regulators of the distinct CDK complexes are also included. See text for more explanations.

Figure 1

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